

EXHIBIT 3

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Making antibody fragments using phage display libraries

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TO BY-PASS hybridoma technology and animal immunization, we are trying to build antibodies in bacteria by mimicking features of immune selection¹. Recently we used fd phage² to display antibody fragments fused to a minor coat protein^{3,4}, allowing enrichment of phage with antigen³. Using a random combinatorial library of the rearranged heavy (V_H) and kappa (V_K) light chains⁵⁻⁸ from mice immune to the hapten 2-phenyloxazol-5-one (phOx), we have now displayed diverse libraries of antibody fragments on the surface of fd phage. After a single pass over a hapten affinity column, fd phage with a range of phOx binding activities were detected, at least one with high affinity (dissociation constant, K_d = 10⁻⁸ M). A second pass enriched for the strong binders at

the expense of the weak. The binders were encoded by V genes similar to those found in anti-phOx hybridomas but in promiscuous combinations (where the same V gene is found with several different partners). By combining a promiscuous V_H or V_K gene with diverse repertoires of partners to create hierarchical libraries, we elicited many more pairings with strong binding activities. Phage display offers new ways of making antibodies from V-gene libraries, altering V-domain pairings and selecting for antibodies with good affinities.

We used the polymerase chain reaction (PCR)⁹ to amplify the V_H and V_K genes from the spleen messenger RNA of mice immunized with phOx, and also developed a 'PCR assembly' process¹⁰ to link these genes together randomly for expression as single-chain Fv (scFv) fragments^{11,12} (Fig. 1a-c). The assembled genes were cloned in a single step into the vector fdDOG1 (Fig. 1e) for display as a fusion with the fd gene III coat protein. This initial library of 2 × 10⁵ clones seemed to be diverse (Fig. 1d), and sequencing revealed the presence of most V_H groups¹³ and V_K subgroups¹⁴ (data not shown). None of the 568 clones tested bound to phOx as detected by enzyme-linked immunosorbent assay (ELISA).

The library of phages was passed down a phOx affinity column (Table 1a), and eluted with hapten. Of the eluted clones, 13%

TABLE 1 Affinity selection of hapten-binding phage

	Precolumn	Clones binding to phOx*		
		After first round	After second round	After third round
(a) Random combinatorial libraries				
phOx-immunized mice	0/568 (0%)	48/376 (13%)	175/188 (93%)	—
Unimmunized mice	—	—	0/388 (0%)	—
(b) Hierarchical libraries				
V _H -B/V _K -rep library	6/190 (3%)	348/380 (92%)	—	—
V _H -rep/V _K -d library	0/190 (0%)	23/380 (7%)	—	—
(c) Fractionation of V _H -B/V _K -d and V _H -B/V _K -b phage*				
Mixture of clones	88/1,896 (4.6%) (44/1,740 (2.5%)†)	55/95 (57.9%)	1,152/1,156 (99.7%)	1,296/1,299 (99.8%)

Selection of phage with hapten-binding activities from the random combinatorial and hierarchical libraries (a and b, respectively), and fractionation of clones with different affinities for phOx (c). For the random combinatorial libraries fdDOG1 RF was extensively digested with *Not*I and *Apal*I, purified by electroelution²⁴ and 1 µg ligated to 0.5 µg (5 µg for the hierarchical libraries) of the assembled scFv genes in 1 ml with 8,000 units T4 DNA ligase (New England Biolabs) overnight at 16 °C. Purified ligation mix was electroporated in six aliquots into MC1061 cells²⁵ and plated on NYZ medium²⁴ with 15 µg ml⁻¹ tetracycline, in 243 × 243 mm dishes (Nunc); 90-95% of clones contained scFv genes by PCR screening (see legend to Fig. 1). Colonies were scraped into 50 ml 2 × TY medium²⁶ and shaken at 37 °C for 30 min. Liberated phage were precipitated twice with polyethylene glycol and resuspended to 10¹² transducing units (TU) ml⁻¹ in water (titred as in ref. 3). For affinity selection, a 1-ml column of phOx-BSA-Sepharose²⁷ (M. Dreher and C. Milstein, unpublished results) was washed with 300 ml PBS, and 20 ml PBS containing 2% skimmed milk powder (MPBS). Phage (10¹² TU) were loaded in 10 ml MPBS, washed with 10 ml MPBS and finally 200 ml PBS. The bound phage were eluted with 5 ml 1 mM 4-*ε*-amino-caproic acid methylene 2-phenyl-oxazol-5-one (phOx-CAP). About 10⁶ TU eluted phage were amplified by infecting 1 ml log phase *E. coli* TG1 (ref. 28) and plating as above. For a further round of selection, colonies were scraped into 10 ml 2 × TY medium and then processed as above. For the hierarchical libraries, V_H-B and V_K-d genes were individually recloned, then assembled with the V_H or V_K repertoires. For the fractionation of clone V_H-B/V_K-d, 7 × 10¹⁰ TU phage in the ratio 20 V_H-B/V_K-b:1 V_H-B/V_K-d were loaded onto a phOx-BSA-Sepharose column in 10 ml MPBS and eluted as above. Eluted phage were used to reinfect *E. coli* TG1, and phage produced and harvested as before. About 10¹¹ TU of phage were loaded onto a second affinity column and the process repeated to give a total of three column passes. Dilutions of eluted phage at each stage were plated in duplicate and probed separately²⁴ with oligonucleotides specific for V_K-b (5'-GAGCGGGTAACCACTGTACT) or V_K-d (5'-GAATGGTATAGTACTACCCCT).

* In (c), numbers refer to V_H-B/V_K-d.

† Numbers after three reinfections and cycles of growth. This control, omitting the column steps, confirms that a spurious growth or infectivity advantage was not responsible for the enrichment of clone V_H-B/V_K-d.

bound to phOx, and ranged from poor to strong binding in ELISA. We sequenced 23 of these hapten-binding clones and found eight different V_H genes (A-H) in a variety of pairings with seven different V_k genes (a-g) (Fig. 2a). Most of the domains, such as V_H -B and V_k -d, were able to bind hapten with any of several partners¹⁵. The probability of finding multiple partners for a given chain should depend mainly on the inherent promiscuity of the chain and on the number of available partners and competing chains. Two other examples of promiscuous pairings have been noted in random combinatorial libraries made in λ phage^{6,8}, so this may prove to be a feature of small combinatorial libraries from immunized animals.

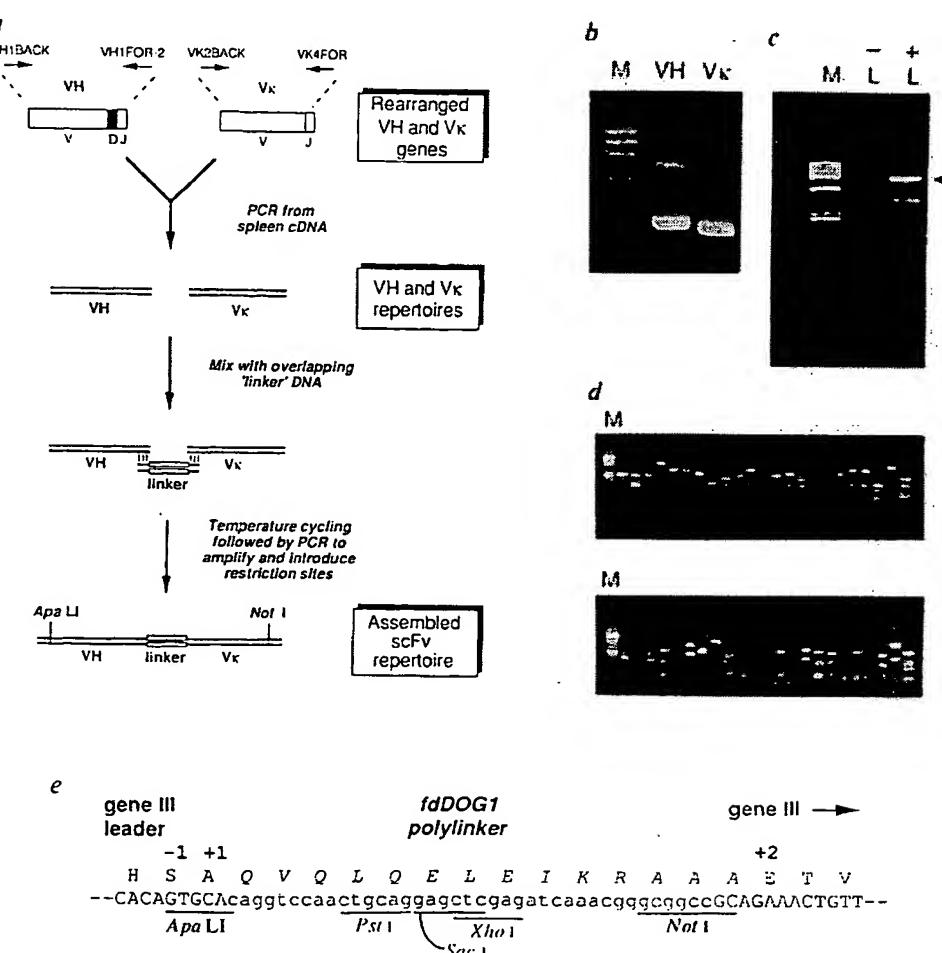
The sequences of the V genes were related to those seen in the secondary response to phOx, but with differences (Fig. 2b). Thus most phOx hybridomas from the secondary response use somatically mutated derivatives of three types of V_k genes, $V_{kox}1$, 'V_{kox}-like' and $V_{k45.1}$ genes¹⁶. These can pair with V_H genes from several groups, but $V_{kox}1$ more commonly pairs with the $V_{Hox}1$ gene (V_H group 2; ref. 13). $V_{kox}1$ genes are

always, and V_{kox} -like genes often, found in association with heavy chain genes (including $V_{Hox}1$) that encode a short five-residue CDR3, with the sequence motif Asp-X-Gly-X-X (where X is any amino acid¹⁶), in which the central glycine creates a cavity for phOx (ref. 17). In our library, nearly all of the V_H genes belonged to group 1, and most of the V_k genes were ox-like and associated with V_H genes encoding a five-residue CDR3 motif Asp/Asn-X-Gly-X-X (Fig. 2b). $V_{kox}1$ and $V_{Hox}1$ were found only once (V_k -f and V_H -E), and not in combination with each other: indeed V_k -f does not encode the Trp 91 involved in phOx binding¹⁷ and was paired with a V_H gene (V_H -C) encoding a six-residue CDR3.

The promiscuity of the V_H -B and V_k -d domains prompted us to force further pairings, by assembling these genes with the entire repertoires of either V_k or V_H genes from the same immunized mice. The resulting hierarchical libraries, (V_H -B \times V_k -rep and V_H -rep \times V_k -d), each with 4×10^7 members, were subjected to a round of selection and hapten-binding clones isolated (Table 1b). Most were strong binders by ELISA

FIG. 1 PCR assembly of scFv library. *a*, V_H and V_k genes are separately amplified, then mixed with a linker fragment that overlaps them both. The linker (93 base pairs) encodes the short peptide, (Gly₂Ser)₃, which links V_H and V_k in scFvs (ref. 11). Cycles of annealing-denaturation, followed by reamplification of the mixture, generate a random combinatorial cassette of V_H and V_k genes joined in-frame for expression. *b*, V_H and V_k gene repertoire PCR products from the immunized mice analysed by electrophoresis on agarose (1%) gel. *c*, PCR assembly of scFv gene repertoires with linker (+L) or without (-L); arrow indicates assembled repertoire. M is DNA marker Φ X174 replicative form DNA digested with *Hae*III. *d*, Diversity of library as seen by *Bst*NI fingerprinting of individual clones. *e*, Sequence of *fd* gene III around the signal peptide cleavage site in *fdDOG1*.

METHODS. For the random combinatorial libraries, cytoplasmic RNA was isolated²⁹ from the pooled spleens of either 5 male BALB/c mice boosted 8 weeks after primary immunization with phOx coupled to chicken serum albumin²⁷, or of 5 unimmunized mice. The cDNA was made with avian myeloblastosis virus reverse transcriptase (Anglian Biotech)³⁰ and primers that straddle the junction between the variable and constant regions of γ heavy chains and κ light chains (C. Marks, unpublished data). V_H and V_k repertoires were amplified from the cDNA with 25 cycles of PCR (94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min) using Vent polymerase (New England Biolabs) and the primers *VH1BACK* (ref. 19) and *VH1FOR-2* (ref. 31) or the primers *VK2BACK* and *VK4FOR*. The linker DNA was similarly amplified from pSW2scD1.3 (ref. 3) using primers *LINKFOR* and *LINKBACK* (complementary to *VK2BACK* and *VH1FOR-2* respectively). After gel purification, 1 μ g each of the V_H and V_k products were mixed with 300 ng linker in a 25 μ l PCR reaction mix without primers, and cycled 7 times (94 °C 2 min, 72 °C 4 min) to join the fragments, then amplified for 20 cycles (94 °C 1.5 min, 72 °C 2.5 min) using 25 pmol each *VH1BACK* and *VK4FOR* primers. Finally, the assembled products were gel-purified and reamplified with *VH1BACK-Apa*I and *VK4FOR-Not*I ('tagged' versions of the original primers) to append restriction sites. Products (1–5 μ g) were extensively digested with *Apa*I



and *Not*I for cloning into *fdDOG1*. Recombinant colonies were screened by PCR³² with the primers *VH1BACK* and *VK4FOR*, followed by digestion with the frequently cutting enzyme *Bst*NI. Primers: *VK2BACK* 5'-GACATTGAGCTC-ACCCAGTCTCCA; *VK4FOR* an equimolar mix of 5'-CCGTTGATTCCACGTT-GGTGCC, 5'-CCGTTTATTCCAGCTGGTCCC, 5'-CCGTTTATTCCAACCTTGT-CCC and 5'-CCGTTTCAAGCTCAGCTGGTCCC; *LINKFOR*, 5'-TGGAGACTGGGT-GAGCTCAATGTC; *LINKBACK*, 5'-GGGACCAAGGTGACCGTCTCCCA; *VH1BACK-Apa*I, as *VH1BACK* (ref. 19) but with 5'-CATGACACAGTCAC added at the 5' end; *VK4FOR-Not*I, as *VK4FOR* but with 5'-GAGTCATTCTCGGGCCG similarly added (restriction sites underlined).

FIG. 2 a, Matrix of V_H and V_k genes identified in phOx-binding clones selected from random combinatorial library. The number of clones found with each combination is shown. The binding to phOx-BSA, as judged by the ELISA signal, seemed to vary (marked by shading): no binding was seen to BSA alone. Optical density at 405 nm: 0.2–0.9, dotted box; 0.9–2.0, hatched box; >2.0, solid box. b, Encoded protein sequences of phOx-binding clones. Sequences of phOx-binding clones isolated (single-letter amino-acid code) after one round of selection of the random combinatorial library, with pairings as above, or the hierarchical library. Note that the first eight or seven residues, and the last nine or eleven residues, of the V_k or V_H genes, respectively, are encoded by the PCR primers. Classifications into V_H groups¹³ and V_k subgroups¹⁴, and the position of residue 91 encoded by the V_k genes (#), are indicated. The relationship to genes from the hybridoma analysed secondary response to phOx (ref. 16) is also shown: all of the V_k genes are 'ox-like', apart from those marked * with an asterisk, which are V_k ox1, and the only example of V_H group 2 (V_H -E) is V_H ox1. The intensity of ELISA signals from the hierarchical libraries, corrected relative to the signal from control phage, are indicated: Optical density at 405 nm 0.9–2.0 (++), >2.0 (+++). Multiple isolations of sequences are marked, and sequences (V_H -B and V_k -c) isolated from the random combinatorial library, and also the hierarchical libraries, are shown in italics. The V_H -B/ V_k -d and V_H -B/ V_k -c pairings gave similar signals (after correction of ELISA) when recovered from either combinatorial or hierarchical libraries.

METHODS. We screened for binding of the phage to hapten by ELISA: 96-well plates were coated with 10 μ g ml⁻¹ phOx-BSA²⁷ or 10 μ g ml⁻¹ BSA in PBS overnight at room temperature. Colonies of phage-transduced bacteria were inoculated into 200 μ l 2 \times TY medium²⁶ with 12.5 μ g ml⁻¹ tetracycline in 96-well plates ('cell wells', Nucleon) and grown with shaking (300 r.p.m.) for

		Heavy chain							
		A	B	C	D	E	F	G	H
Light chain	a	2		1					
	b		1		1	1			
	c		1						1
	d		7	1				1	
	e	2			2				
	f			1					
	g								1

24 h at 37 °C. At this stage, cultures were saturated and phage titres were reproducible (10^{10} TU ml⁻¹). Phage supernatant (50 μ l), mixed with 50 μ l PBS containing 4% skimmed milk powder, was then added to the coated plates. Further details given in ref. 3. To sequence the clones, template was prepared²⁴ from the supernatants of 10-ml cultures grown for 24 h, and sequenced using the dideoxy method³³ and a Sequenase kit (USB), with primer LINKFOR for the V_H genes and primer fdSEQ1 (5'-GAATTTCTGTATGAGG) for the V_k genes.

b

 V_H sequences

From combinatorial library:

	CDR1	CDR2	CDR3	Group	ELISA signal
a	OVLQGSQELARPGAVKMSCKASGYTT	SYTMH WVKQRPQGQLEWIG YINPSSGTYTNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR RYGY	WCGQTTTVVSS	x4
b	OVLQGSQELARPGAVKMSCKASGYTT	RDMMH WVKQRPQGQLEWIG YINPSTGTYTEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR NYGLY	WCGQTTTVVSS	x9
c	OVLQGSQELARPGAVKMSCKASGYTT	SYVNH WVKQRPQGQLEWIG YINPYDGTQYNEKFKG	KATLTADKSSSTAYMELSSLTSESDAVYYCAR YRSFPY	WCGQTTTVVSS	x3
d	OVLQGSQELARPGAVKMSCKASGYTT	GYFMH WVKOSHGSLEWIG RINPYNGDTEYNQNKFKG	KATLTADKSSSTAYMOLSSLTSESDAVYYCVO ITTRFAY	WCGQTTTVVSS	x3
e	OVLQESGPGLVVAQSPQLSITCTVGSFL	SYGWH WVKQRPQGQLEWIG YINWAGGSNTYNSALMRS	RLS15KDNKSOVFLKMNLSLTDDTAMYYCAR DRGYD	WCGQTTTVVSS	1 (see Fig. 2a)
f	OVLQGSQELARPGAVKMSCKASGYTT	SYLMH WVKQRPQGQLEWIG YINPSTGTYTEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1
g	OVLQGSQELARPGAVKMSCKASGYTT	SYLMH WVKQRPQGQLEWIG YINPSTGTYTEYNQNKFKD	EATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1
h	OVLQGSQELARPGAVKMSCKASGYFS	RYNMH WVKOSHGSLEWIG YIAFPNGGTYTEYNQNKFKG	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1

From hierarchical library V_H -rep \times V_k -rep:

I	OVLQGSQELARPGAVKMSCKASGYTT	SYAMH WVKQSOQSLSLEWIG VISTYNGNTYNYQNKFKG	KATMTVDKSSSTAYMELARLTSESDAIYYCAR DYGYY	WCGQTTTVVSS	1	+++
J	OVLQGSQELARPGAVKMSCKASGYTT	RYTMH WVKQRPQGQLEWIG YINPSTGTYTEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DRGY	WCGQTTTVVSS	1	+++
K	OVLQGSQELARPGAVKMSCKASGYTT	RDMMH WVKQRPQGQLEWIG YINPSTGTYTEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR NYGLY	WCGQTTTVVSS	x3	+++
L	OVLQGSQELARPGAVKMSCKASGYTT	NYLMMH WVKQRPQGQLEWIG YINPSTGTYTEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1	++
M	OVLQGSQELARPGAVKMSCKASGYTT	NYMMH WVKQRPQGQLEWIG YINPSTGTYTEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1	++
N	OVLQGSQELARPGAVKMSCKASCEASYG	SHLMH WVKQRPQGQLEWIG YINPRTGTYEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1	++
O	OVLQGSQELARPGAVKMSCKASGYTT	SYLMMH WVKQRPQGQLEWIG YINPSTGTYEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1	++
P	OVLQGSQELARPGAVKMSCKASGYTT	SYMMH WVKQRPQGQLEWIG YINPSTGTYEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1	++
Q	OVLQGSQELARPGAVKMSCKATGYTT	SYLMMH WVKQRPQGQLEWIG YINPSTGTYEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1	++
R	OVLQGSQELARPGAVKMSCKASGYTT	SYVMH WVKQRPQGQLEWIG YINPSSGTYTNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR NYGLY	WCGQTTTVVSS	1	++
S	OVLQGSQELARPGAVKMSCKASGYTT	TFLMH WLRKQRPQGQLEWIG YINPSTGTYEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	x2	+++
T	OVLQGSQELARPGAVKMSCKASGYTT	SYTHM WVKQRPQGQLEWIG YINPSSGTYTNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	x6	+++
U	OVLQGSQELARPGAVKMSCKASGYTT	SYTHM WVKQRPQGQLEWIG YINPSTGTYEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR DYGYY	WCGQTTTVVSS	1	+++
V	OVLQGSQELARPGAVKMSCKASGYTT	RDMMH WLRKQRPQGQLEWIG YINPSTGTYEYNQNKFKD	KATLTADKSSSTAYMOLSSLTSESDAVYYCAR NYGLY	WCGQTTTVVSS	1	+++

 V_k sequences

From combinatorial library:

	CDR1	CDR2	CDR3	Group	ELISA signal	
a	DIELTOSPSSLSASLGERVSLTC	RASQEIISGYLS	WLOQPKDGSIKRLLY	AASLTS	WCGQTTTVVSS	x3
b	DIELTOSPAIMSASPEKVTMTC	RASSSVSSYLY	WYQOKSGASPKVWIIY	STSNLAS	WCGQTTTVVSS	IV
c	DIELTOSPITAASPEKIIITC	SASSSISNNYLY	WYQOKPGFSPKLLIY	RTSNLAS	WCGQTTTVVSS	(see Fig. 2a)
d	DIELTOSPITAASPEKIIITC	SASSSISNNYLY	WYQOKPGFSPKLLIY	RTSNLAS	WCGQTTTVVSS	IV
e	DIELTOSPAIMSASPEKVTMTC	SASSSVNYYM	WYQOKPGTSPKRWIY	DTSKLAS	WCGQTTTVVSS	VI
f	DIELTOSPAIMSASPEKVTMTC	SASSSVSYNM	WYQOKSGTSPKRWIY	DTSKLAS	WCGQTTTVVSS	VI
g	DIELTOSPAIMSASPEKVTMTC	SASSSINYMH	WYQOKPGASPKRWIY	DTSKLAS	WCGQTTTVVSS	VI

From hierarchical library V_H -B \times V_k -rep:

b	DIELTOSPAIMSASPEKVTMTC	SASSSVSYMH	WYQOKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTYSLSLTISSEADAATYYC	QWQSNPLT	FGAGTKEIKRA	x4	VI	+++
i	DIELTOSPAIMSASPEKVTIITC	SASSSVSYIH	WFOQKPGTSPKRWIY	STSNLAS	GVPARFSGSGSGTYSLSLTISRMEEADAATYYC	QWQHSPPLT	FGAGTKEIKRA	VI	+++	
j	DIELTOSPITAASPEKIIITC	SASSSISNNYLY	WFOQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTYSLSLTIGTMEADAATYYC	QGSSIPPLT	FGAGTKEIKRA	x2	IV	++
k	DIELTOSPITAASPEKIIITC	SASSSISNNYLY	WFOQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTYSLSLTIGTMEADAATYYC	QGSSIPPLT	FGAGTKEIKRA	x9	IV	(see Fig. 2a)
l	DIELTOSPITAASPEKIIITC	SASSSISNNYLY	WYQOKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTYSLSLTIGTMEADAATYYC	QGSSIPPLT	FGAGTKEIKRA	x4	VI	++
m	DIELTOSPITAASPEKIIITC	SASSSISNNHLY	WYQOKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTYSLSLTIGTMEADAATYYC	QGSSIPPLT	FGAGTKEIKRA	IV	++	
n	DIELTOSPITAASPEKIIITC	SASSSISNNYLY	WYQOKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTYSLSLTIGTMEADAATYYC	QGSSIPPLT	FGAGTKEIKRA	IV	++	
o	DIELTOSPITAASPEKIIITC	SASSSISNNYLY	WYQOKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTYSLSLTIGTMEADAATYYC	QGSSIPPLT	FGAGTKEIKRA	x2	IV	++
p	DIELTOSPAIMSASPEKVTMTC	SASSSVSYMH	WYQOKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTYSLSLTISSEADAATYYC	QWQSNPLT	FGAGTKEIKRA	x2	VI	+++
q	DIELTOSPAIMSASPEKVTMTC	SASSSVSYMH	WYQOKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTYSLSLTISSEADAATYYC	QWQSNPLT	FGAGTKEIKRA	x2	VI	+++
r	DIELTOSPAIMSASPEKVTMTC	SASSSVSYMH	WYQOKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTYSLSLTISSEADAATYYC	QWQSNPLT	FGAGTKEIKRA	VI	++	
s	DIELTOSPAIMSASPEKVTMTC	RASSSVSSYLN	WYQOKSGASPKLWIIY	STSNLAS	GVPARFSGSGSGTYSLSLTISSEADAATYYC	QWQHSPPLT	FGAGTKEIKRA	IV	++	
t	DIELTOSPAIMSASPEKVTMTC	RASSSVSSYLN	WYQOKSGASPKLWIIY	STSNLAS	GVPARFSGSGSGTYSLSLTISRMEEADAATYYC	QWQSYPLT	FGAGTKEIKRA	IV	++	
u	DIELTOSPAIMSASPEKVTMTC	RASSSVSSYLN	WYQOKSGASPKLWIIY	STSNLAS	GVPARFSGSGSGTYSLSLTISRMEEADAATYYC	QWQSYPLT	FGAGTKEIKRA	IV	++	
v	DIELTOSPAIMSASPEKVTMTC	RASSSVSSYLN	WYQOKSGASPKLWIIY	STSNLPS	GVPARFSGSGSGTYSLSLTISSEADAATYYC	QWQSYPLT	FGAGTKEIKRA	IV	++	
w	DIELTOSPITAASPEKIIITC	SASSSISNNYLY	WYQOKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTYSLSLTIGTMEADAATYYC	QGSSIPPLT	FGAGTKEIKRA	x3	IV	++

(Fig. 2b). By sequencing 23 clones from each library, we identified 14 new partners for VH-B and 13 for V κ -d; apart from VH-B and V κ -c, none of the previous VH-B or V κ -d partners (or indeed other partners) cloned and sequenced from the random combinatorial library was isolated again. These features are consistent with the much larger number of available partners (4×10^7) for the VH-B (or V κ -d) domain, and the promiscuous nature of the domain. The V κ genes were mainly ox-like and the VH genes mainly group 1, but the only examples of V κ ox1 (V κ -h, -p, -q and -r) encode Trp 91, and the VH-CDR3 motif Asp-X-Gly-X-X now predominates. Thus some features of the phOx hybridomas seem to emerge more strongly in the hierarchical library. The new partners differ from each other mainly by small alterations in the CDRs, indicating that much of the subtle diversity had not been tapped by the original random combinatorial library. More generally we find that a range of related antibodies can be made by keeping one of the partners fixed and varying the other, and this could be invaluable for fine tuning of antibody affinity and specificity.

To determine the range of antibody affinities for phOx, we recloned the combinations of VH-B with V κ -b and V κ -d (which gave weak and strong binding signals to phOx in ELISA) for secretion as soluble scFv fragments (Fig. 3, legend). Fluorescence quench titrations determined the K_d of VH-B/V κ -d for phOx-GABA as 1.0×10^{-8} M (Fig. 3a), indicating that antibodies with affinities representative of the secondary response can be selected from phage display libraries. Indeed of anti-phOx hybridomas from the secondary response, only two (out of 11 characterized) secrete antibodies of a higher affinity than VH-B/V κ -d (ref. 16). The K_d of VH-B/V κ -b for phOx-GABA was determined as 1.8×10^{-5} M (Fig. 3b); thus phage bearing

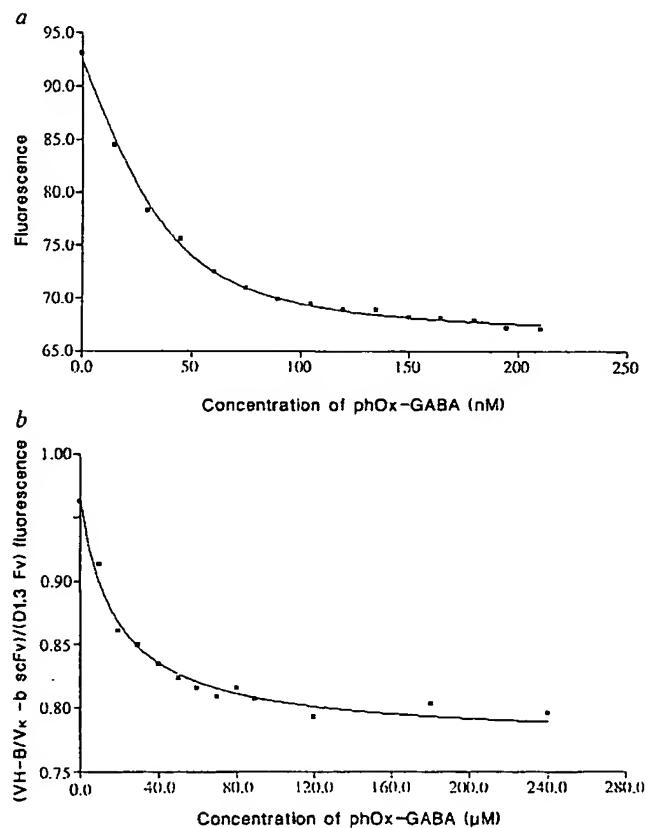
scFv fragments with weak affinities can also be selected with antigen, probably because of the avidity of the multiple antibody heads on the phage.

A second round of selection of the original, random combinatorial library from immune mice resulted in 93% of eluted clones binding phOx (Table 1a). Most of these clones were V κ -d combinations, and bound strongly to phOx in ELISA (data not shown). Few weak binders were seen. This suggested that affinity chromatography had not only enriched for binders, but also for the best. To confirm this we mixed the phage VH-B/V κ -d with a 20-fold excess of the phage VH-B/V κ -b and subjected the mixture to rounds of selection: after only two rounds, essentially all the eluted phage were VH-B/V κ -d (Table 1c).

We also constructed a random combinatorial library (2×10^6 members) from unimmunized mice, but found no phOx-binding clones after two rounds of selection (Table 1a). Immunization therefore seems to be necessary to create and/or enrich for VH or V κ domains with at least some of the features required for hapten binding. With libraries of this size ($\sim 10^6$ members), such domains need to be represented at a high frequency to reconstitute a binding site¹, and immunization ensures this by biasing the spleen lymphoid cell population heavily towards messenger RNA-rich blast cells making specific antibody (R. Hawkins and G.W., unpublished data). With larger libraries ($> 10^7$) now accessible using selection³ rather than screening⁶⁻⁸, immunization may be unnecessary for the isolation of antibody fragments. It has been estimated that a library of 10^7 different antibodies will probably recognize $> 99\%$ of epitopes with a dissociation constant of $\geq 10^{-5}$ M (ref. 18), and we have shown here that we can recover antibody fragments with such affinities

FIG. 3 Fluorescence quench titration of soluble scFv fragments. a, The K_d ($1.0 \pm 0.2 \times 10^{-8}$ M) for clone VH-B/V κ -d was determined by fluorescence quench titration³⁴ of purified scFv (100 nM) with 4- γ -amino-butyric acid methylene 2-phenyl-oxazol-5-one (phOx-GABA). Excitation was at 280 nm, emission was monitored at 340 nm and the K_d calculated as in refs 35 and 36. All values were calculated with standard errors included. The K_d was determined to be $1.0 \pm 0.2 \times 10^{-8}$ M with 0.38 ± 0.05 binding sites per scFv molecule. b, For measurement of the K_d of the low affinity clone VH-B/V κ -b, 2 μ M purified scFv protein was titrated with phOx-GABA as above. But to minimize light absorption by the higher concentrations of phOx-GABA required, excitation was at 260 nm and emission was monitored at 304 nm. In addition, the fluorescence values were divided by those from a parallel titration of the lysozyme binding Fv fragment D1.3 (ref. 31). The K_d was calculated as described in refs 34 and 36 and determined to be $1.8 \pm 0.3 \times 10^{-5}$ M, with a fractional quench of 0.20 ± 0.01 .

METHODS. Clones VH-B/V κ -b and VH-B/V κ -d were reamplified with VK4FOR-NotI and VH1BACK-SfiI (5'-CATGCCATGACTCGGGCCCAGCCGGCATGG-CC(G/C)AGGT(C/G)(A/C)(A/G)CTGCAG(C/G)AGTC(A/T)GG-3'), a primer that introduces an SfiI site (underlined) at the 5' end of the VH gene. VH-B/V κ -d was cloned into the phagemid pJM1 (A.D.G. and J. Marks, unpublished results) as an SfiI-NotI cassette, downstream of the pelB leader for periplasmic secretion³⁷, with a C-terminal peptide tag for detection^{31,38}, and under the control of a λ P_L promoter³⁹. Cultures (10 l) of *Escherichia coli* N4830-1 (ref. 40) harbouring each phagemid were induced²⁶ and supernatants precipitated with 50% ammonium sulphate. The resuspended precipitate was dialysed into PBS, 0.2 mM EDTA (PBSE), loaded onto a 1.5-ml column of phOx-Sepharose⁴¹ and the column washed sequentially with 100 ml PBS; 100 ml 0.1 M Tris-HCl, 0.5 M NaCl pH 8.0; 10 ml 50 mM citrate, pH 5.0; 10 ml 50 mM citrate, pH 4.0 and 20 ml 50 mM glycine, pH 3.0. The scFv fragment was eluted with 50 mM glycine, pH 2.0, neutralized with Tris base and dialysed against PBSE. VH-B/V κ -b was cloned into a phagemid vector (A.D.G., unpublished results) based on pUC119 (ref. 42) encoding identical signal and tag sequences to pJM1, and expression induced at 30 °C in a 10-culture of *E. coli* TG1 (ref. 28) harbouring the phagemid, as in ref. 43. The low affinity of clone VH-B/V κ -b made its purification on phOx-Sepharose impossible. Therefore after concentration by ultrafiltration (Filtron, Flowgen) the supernatant (100 ml of 600 ml) was loaded onto a 1-ml column of protein A-Sepharose coupled⁴⁴ to the monoclonal antibody 9E10 that recognizes



the C-terminal peptide tag^{31,38}. The column was washed with 200 ml PBS and 50 ml PBS, 0.5 M NaCl. The scFv fragment was eluted with 100 ml 0.2 M glycine, pH 3.0, with neutralization and dialysis as before.

from phage display libraries. The antibody fragments could be rebuilt from their genes into complete antibodies, and expressed in myeloma cells if required, as described in ref. 19.

It may be possible to retain the original V_H/V_L pairings of the splenocytes, as in hybridoma technology. In principle, PCR assembly could be used to construct such 'natural' libraries, if the V genes from individual cells could be amplified and assembled in capsules. More immediately, affinity selection from combinatorial and hierarchical libraries promises an attractive route to high-affinity antibodies, in particular those from humans that are difficult to produce by hybridoma technology¹. But the use of phage display libraries is not limited to antibodies: it offers a powerful and general method to change and refine the properties of any other protein²⁰ or peptides²¹⁻²³ that can be displayed on the phage surface. □

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Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene

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A CYCLIC AMP-stimulated chloride conductance appears when the cystic fibrosis gene is expressed in non-epithelial cells by infection with recombinant viruses^{1,2}. Cyclic AMP-stimulated conductance in this system is mediated by the same ohmic, low-conductance Cl^- channel as in human secretory epithelia²⁻⁴, but control of this channel by phosphorylation has not been directly demonstrated. Here we report the appearance of the low-conductance Cl^- channel in Chinese hamster ovary cells after stable transfection with the cystic fibrosis gene. The channel is regulated on-cell by membrane-permeant analogues of cAMP and off-cell by protein kinases A and C and by alkaline phosphatase. These results are further evidence that the cystic fibrosis transmembrane regulator is a Cl^- channel which can be activated by specific phosphorylation events and inactivated by dephosphorylation; they reveal an unsuspected synergism between converging kinase regulatory pathways.

The coding sequence of the cystic fibrosis transmembrane regulator (CFTR) was cloned behind the metallothionein pro-

moter of a plasmid that also contained a mutant dihydrofolate reductase gene, driven by the simian virus 40 early promoter (Fig. 1a). Stably transformed colonies were selected with methotrexate after calcium phosphate transfection of Chinese hamster ovary (CHO)-K1 cells. CFTR-expressing variants were chosen for further study on the basis of their capacity to produce a protein of the same apparent size as that present in T84 cell membranes in western blots probed with monoclonal antibodies against CFTR (Fig. 1b). CFTR protein was localized in a highly enriched plasma membrane vesicle fraction. In variants containing nearly the same amount of CFTR as T84 cells, cAMP-regulated chloride permeability, as monitored by ¹²⁵I efflux, was indistinguishable from that in T84 cells (Fig. 1c).

Patch-clamp recording was used to identify the channel responsible for cAMP-stimulated ¹²⁵I efflux. Channels became active in cell-attached patches after a lag of 69 ± 26 seconds when cells were exposed to membrane-permeant derivatives of cAMP; this was reversed by washing cAMP from the bath ($n = 7$, Fig. 2a). The channel was observed in 80% of all seals during cAMP stimulation (225/282) at an average density of between five and ten channels per patch. By contrast, it was recorded only once in 55 patches on unstimulated, CFTR-transfected cells and was never observed on cAMP-stimulated CHO cells that had been transfected with vector alone (0/31). Figure 2b shows that open probability was relatively independent of voltage, despite increased flickering at hyperpolarized potentials. Flickering was not observed using excised patches (see below), therefore these brief closures may reflect voltage-dependent, fast channel block by some anion in the cytosol. The current-voltage relationship rectified slightly in the outward direction during cell-attached recordings (Fig. 2c), but was linear ($r^2 = 0.9997$) when patches were excised and bathed symmetrically with 154 mM Cl^- (data not shown). In cell-attached patches the reversal potential (E_{rev}) was near the membrane potential (0.6 ± 0.3 mV applied potential) and the slope conductance at E_{rev} was 9.6 ± 0.5 pS ($n = 5$). The E_{rev} shifted to $+32.4 \pm 2.0$ mV when the pipette solution contained 110 mM sodium gluconate and

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